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by

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ABSTRACT

Johnson, A., Binette, S.L., Cook-Versloot, M., Beattie, M., McGeachy, S., Gagné, N., McDonald, J.T, and Ritchie, R.J. 2008 Association between ISAV mortalities and ISAV molecular type in the Bay of Fundy, Canada. Can. Tech. Rep. Fish. Aquat. Sci. 2782:iv+15pp.

Since Infectious Salmon Anaemia was first detected in the Bay of Fundy over a decade ago, it has been a primary focus of fish health management efforts. The causative agent, the Infectious Salmon Anaemia virus (ISAV) has been well characterized at the molecular level. Numerous genetically distinct ISAV isolates have been identified and molecular typing is routinely used to track the pathogen. Several epidemiological studies have identified risk factors for pathogen transmission and disease progression prompting development of new management practices. However, ISAV remains a threat to aquaculture stock and a concern to salmon farmers. Significant anecdotal data exists for the presence of low and high virulent strains which likely differ in mortality, disease progression and infectivity. These anecdotal findings are supported by *in vitro* studies which show that different molecular types have different virulence. As management of ISAV often relies on eradication of infected cages, it would be of immense benefit to management to validate these findings in the field. The goal of this study was to investigate molecular variation of the ISAV variable region on ISAV segments 5 and 6 in a panel of field isolates, and to compare this data to recorded field mortalities. Our field work completed in the winter of 2005/06 identified three main HPR types (and associated amino acid variants), and suggests a correlation between HPR type and recorded mortality levels in the field.

KEY WORDS: Infectious salmon anemia virus, ISAV, HPR, haemaglutinnin-esterase, mortality, sequencing

RÉSUMÉ

Johnson, A., Binette, S.L., Cook-Versloot, M., Beattie, M., McGeachy, S., Gagné, N., McDonald, J.T, and Ritchie, R.J. 2008 Association between ISAV mortalities and ISAV molecular type in the Bay of Fundy, Canada. Can. Tech. Rep. Fish. Aquat. Sci. 2782:iv+15pp.

Depuis la première apparition de l'anémie infectieuse du saumon (AIS) dans la Baie de Fundy il y a plus de 10 ans, la gestion de la santé des poissons de

cette région s'est principalement concentrée sur ce problème. L'agent responsable de la maladie, le virus de l'AIS, est maintenant mieux connu au niveau moléculaire. De nombreuses souches distinctes du virus ont été identifiées et le typage moléculaire est utilisé couramment pour suivre l'évolution du pathogène. Plusieurs études épidémiologiques ont identifié des facteurs de risque dans la transmission du pathogène et la progression de la maladie, amenant également le développement de nouvelles pratiques de gestion. Toutefois, le virus de l'AIS continue de menacer les stocks d'aquaculture et d'inquiéter les aquaculteurs. Plusieurs observations sur le terrain semblent aussi indiquer qu'il y aurait des différences significatives dans les taux de mortalité en fonction de la souche virale; ainsi il y aurait des souches hautement virulentes et d'autres peu virulentes. Ces observations sont supportées par des études de laboratoires contrôlées démontrant que des souches spécifiques ont des virulences significativement différentes. Puisque la gestion de l'AIS comprend souvent l'éradication d'une cage où l'infection est diagnostiquée, il serait bénéfique de valider ces observations concernant la virulence plus ou moins forte de certaines souches au niveau de l'aquaculture. Le but de cette étude est d'étudier la variation moléculaire du virus de l'AIS au niveau du segment 5 et 6 chez des souches isolées sur le terrain, et de comparer ces données avec les taux de mortalités occasionnés par ces mêmes souches sur le terrain. Ces échantillons et observations furent amassés à l'hiver 2005/2006 et ont permis d'identifier 3 principaux génotypes liés au segment 6 ou région hypervariable (HPR), et suggèrent une corrélation entre le type d'HPR et le taux de mortalité observé sur le terrain.

MOTS-CLÉS : anémie infectieuse du saumon, AIS, HPR, hémagglutinine-estérase, mortalités, séquençage

INTRODUCTION

Infectious salmon anemia is a serious disease of Atlantic salmon and one which has caused, and continues to cause, significant economic losses in the form of direct costs and depopulation losses to salmon farming industries in Norway (Thorud and Djupvik, 1988), Scotland (Rodger et al., 1998; Rowley et al., 1999), Maine (Bouchard et al., 2001), Chile (Kibenge et al., 2001a) and on the Canadian East Coast (Mullins et al., 1998; Lovely et al., 1999; Blake et al., 1999; Bouchard et al., 1999). The disease is associated with varying levels of mortality and clinical symptoms including severe anaemia, congestion of the liver, spleen and foregut and haemorrhagic liver necrosis (Thorud and Djupvik, 1988; Evensen et al., 1991). In 2006 Infectious Salmon Anaemia was listed by the Office International des Epizooties (OIE) and on the east coast of Canada it remains the focus of fish health management programs established to monitor the disease and infectious agent (OIE, 2006a; Ibid, 2006b).

Several epidemiological studies have been carried out in Eastern Canada and in other areas affected by ISA in order to identify risk factors associated with ISA outbreaks (Wheatley et al., 1995; Scheel et al., 2007, Murray et al., 2002; Hammel and Dohoo 2005a; Ibid, 2005b; Gustafson et al., 2007a; Nylund et al., 2007). These studies concur that vessel traffic, holdover farms, biomass and an infected neighboring farm are risk factors. On the east coast of Canada, sharing of equipment or personnel, type of feed, feed delivery methods, multi-year class sites, stress and husbandry procedures, density of fish/water volume and recent infections on upstream farms have also been identified as risk factors, although not all studies agree on these findings (Gustafson et al., 2007b, Hammell et al., 2005b). Nevertheless, these studies have helped to improve cage site biosecurity and to redefine Bay Management Areas (BMA) as part of an overarching fish health management plan, and appear to reduce the economic impact of ISA on salmon farming operations.

The causative agent, Infectious Salmon Anaemia virus (ISAV) is a segmented, negative stranded RNA orthomyxovirus, and has been well characterized at the molecular level (Mjaaland et al., 1997; Krossøy et al., 1999; Ritchie et al., 2001a; Krossøy et al., 2001a; Rimstad et al., 2001; Kristiansen et al., 2002; Ritchie et al., 2002; Cunningham and Snow, 2000; Clouthier et al., 2002; Falk et al., 2004; Hellebø et al., 2004). Comparative nucleotide sequence analysis of ISAV isolates from Europe and North America indicate the existence of two broad clades, estimated to have diverged between 100 and 200 years ago (Krossøy et al., 2001b; Nylund et al., 2003). The continental clades are not

geographically discrete, as infections caused by virus with nucleotide sequences typical of European virus have been found in North America (Ritchie et al., 2001b; Kibenge et al., 2001b). Sequencing of isolates from both continents has revealed significant nucleotide variation on segment 6 (Krossøy et al. 2001a; Rimstad et al. 2001; Kibenge et al. 2001b; Devold et al. 2001; Griffiths et al. 2001) and on segment 5 (Devold et al. 2006; Ritchie unpublished). Variation on the latter site encoding the fusion protein, along with synteny to the virulence proteins in human influenza makes it a candidate virulence factor. However, its role in virulence has yet to be studied in detail. By contrast the variation on segment 6 encoding the haemaglutinnin (HA) protein has been relatively well characterized and variation in this hypervariable region (HPR) is now routinely used to 'type' isolates to monitor the spread of the infection (McGeachy, personal communication). Approximately 25 different variants of the ISAV HA HPR have been found, along with a larger 'archetype' sequence termed HPR0 (Cunningham et al., 2002; Nylund et al., 2003; Mjaaland et al., 2002; Cook-Versloot et al., 2004). The HPR0 is believed to be avirulent and the progenitor of shorter European HA HPR variants (Cunningham et al., 2002), although it is important to note that the shorter exist in the Bay of Fundy are presumed to arise from a North American HPR0 homology which has not yet been identified. Indeed the relationship between different HPR genotypes and disease in the field has not been well characterized for either the shorter North American or European HA HPR variants.

There is significant anecdotal evidence that the HPR on segment 6 is associated with differences in manifestation of disease or infectivity. Phenotypic differences have been observed in cell culture with ISAV isolates of different molecular type showing different affinities to different cell lines (Kibenge et al., 2000; Griffiths et al., 2001). Furthermore, *in vivo* studies of ISAV isolates possessing different HPR types have shown different levels of mortality and differences in disease progression (Kibenge et al., 2006; B. Glebe et al., unpublished). Thus, it would be useful to management of ISAV if it were possible to characterize or delineate differences in disease severity or progression in field samples associated with different ISAV molecular types, especially for management schemes involving depopulation of infected cages.

To date no field studies comparing molecular type and disease progression have been published and virulence levels of individual ISAV isolates in the Bay of Fundy have not been formally studied. The goal of this study was to type the segment 6 HPR and segment 5 variable regions in a panel of ISAV field isolates, and correlate this molecular typing data with mortalities associated

with each sample site to determine whether variation at either locus correlates with virulence. Our study includes all ISAV positive cages in the Bay of Fundy during the winter 2005/06 and includes two year classes of fish and a range of sites from a broad geographic region.

MATERIALS AND METHODS

SAMPLE AND DATA COLLECTION

Twelve to 18 months old moribund or fresh dead fish found during routine bi-weekly or monthly surveillance were transported to the lab and necropsied. Small pieces of kidney were used to make imprints for IFAT, and archived in RNAlater (Ambion) for diagnostic ISAV RT-PCR. . Samples testing positive for ISAV either through IFAT, RT-PCR or both, were selected for typing of the HPR of segment 6 (total of 108 samples) and the putative variable region of segment 5.

Mortality information (number of mortalities per cage) for each cage/cage site was collected bi-weekly, weekly, or bi-monthly, by site managers and compiled in an Excel spreadsheet. Other potential causes for gross mortalities, such as seals, other diseases such as Bacterial Kidney Disease (BKD), skin lesions, or storms, were also recorded.

RNA EXTRACTION AND RT-PCR

Total RNA was extracted from kidney tissue stored in RNAlater, using Trizol Reagent (Gibco, BRL) as per manufacturer's instructions and suspended in 50 µl DEPC-treated water. Previously extracted RNA was received frozen at a concentration of ~1000 ng/µl. The HPR of ISAV segment 6 was amplified with the Qiagen One-Step RT-PCR system (Qiagen) using the manufacturer's protocol with the following three primers in one reaction: HAFnew-NA1 5' TTGTGAAAGAATTTGACCAAACA 3', HAFnew-NA2 5' TGGTTAAAGATTTTGACCAGACA 3', and 1414mod-NA 5' ACAGAGCAATCCCAAAACCTG 3'. Briefly, 2 µg of total RNA from kidney tissue RNA extracts was reverse transcribed and then PCR amplified in a total volume of 50 µl with primer concentrations of 0.6 µM. The RT-PCR conditions were as follows: 50°C for 30 min, 95°C for 15 min, followed by 40 cycles of 94°C for 30 s, 62°C for 45 s, and 72°C for 1 min, then a final extension of 72°C for 10 min.. For amplification of the segment 5 putative variable region, the degenerate primers Seg5var-F1 5'-GTACCWGACARGCTAGGGTT, Seg5var-R1 5'-CAAGGWGACGGTGCMGCATC-3' were used and the annealing temperature was 55°C for 45 s.

GEL ELECTROPHORESIS AND PCR PRODUCT PURIFICATION

Samples were mixed with loading buffer and were run on 11% acrylamide gels at 200V for 1 hr to check for PCR yield prior to sequencing. Amplified DNA from each sample was then purified using QIAquick PCR purification kit (Qiagen) and eluted in 30-50 µl of elution buffer (Qiagen).

SEQUENCING ANALYSIS

Depending on amplicon strength, 1-10 µl of purified PCR product was combined with 3.2 pmol sequencing primer, 4 µl of Big Dye Terminator solution (Applied Biosystems) and 4 µl Big Dye 5X Sequencing buffer (Applied Biosystems) in a total reaction volume of 20 µl and cycled 25 times at 96°C for 10 s, 50°C for 5 s, and 60°C for 4 min. The primers used for sequencing were the same used for RT-PCR. After cycling, the reactions were purified with Edge Biosystem gel filtration cartridges (Bio-Rad) according to manufacturer's instructions. The eluted product was heated at 93°C for 3 minutes and snap cooled on ice for at least 3 minutes. Samples were then electrophoresed on an ABI3100 Genetic Analyser (Applied Biosystems) and further analyzed using Sequencher software (Genecodes).

STATISTICAL ANALYSIS

In order to obtain an estimate of pathogen virulence in the cages associated with each sample, mortality levels for each cage were quantified for a set period of time before the sampling date. Mortality records from 62 cages from 17 sites were made available by the site managers and were included in the study. Cages for which mortality data was not provided were not included. Approximate percent mortalities were calculated for the month prior to when each sample was collected. These values were considered to represent mortality levels due to ISAV around the time of sampling. In some cases information on other causes of mortality such as other disease (e.g. BKD), storm damage and seal attacks was provided. This information when available, along with year class, time and location of stocking, was included in the subsequent statistical analyses.

The specific focus of the statistical analysis was on the logarithm of the mortality rate, computed as the mortality level in a particular cage observed in the month prior to the sampling period divided by the number of fish initially present

in the cage. The relationship between the type of ISAV strain present and mortality rate was estimated using ordinary least squares (OLS) regressions in which the dependent variable was the log mortality rate and the regressors included indicator variables for ISAV strain as well as indicator variables for various other potentially confounding factors. In order to gauge the impact of including confounding factors on the estimated relationship between ISAV strain and mortality rate, a series of regression models were estimated. As a benchmark, Model 1 controlled only for ISAV strain by including separate indictor variables in the model for ISAV2, ISAV4, and untyped ISAV, all measured against the default category ISAV0. Model 2 added an indicator variable for year class (if the cage was stocked in 2005, with 2004 the default category), plus a set of indicator variables for certain events that had a significant impact on mortality but were unrelated to ISAV.[2] Model 3 added indicator variables for site location, in two different ways. Model 3a included a simple indicator variable for whether the site was in the Northern part of the Bay of Fundy (the default category is the southern part), while Model 3b instead included a set of three indicator variables to differentiate among the four bay management areas (BMA) from which cage data were draw. This model based on BMA captures additional geographic details such a tidal excursions.

Data were analysed using STATA v10.0. Once OLS results were obtained, the resulting estimates were then used to generate predicted mortality rates by ISAV strain under a range of alternative scenarios that hold constant the possible impact on mortality rate of the various confounding factors controlled for in the analysis. The statistical significance of differences in mortality rates across ISAV strains was determined using standard methods.

RESULTS

SAMPLE COLLECTION AND MOLECULAR TYPING

A total of 108 samples collected between January and March 2006, shown to be positive for ISAV using segment 8-based RT-PCR and/or IFAT were used

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[2] Specifically, an indicator variable was included to reflect the occurrence of seal attacks that caused significant mortality in certain cages during the sampling period. As noted by a referee, seal attacks and ISAV infection may well be related. For example, it may be possible that seal attacks may arise because of higher mortality from ISAV infection. We will examine this possibility in future work. Also, in results not reported here, other variables were also included to reflect mortality caused by BKD and by skin lesions arising from poor weather. The inclusion of these variables had little effect on any of the results reported in the tables and figures.

in reactions to amplify the HPR of segment 6 and the variable region of segment 5. The 108 samples represented 26 different cage sites including sites in Deer Island, Lime Kiln Bay and Grand Manan, and were considered to be representative of ISAV infections across the industry at the time of the study (McGeachy, personal communication). In some cases, multiple samples from the same site or cage were collected in the preliminary screening. Eighty one (81) and seventy nine (79) of these samples produced PCR amplicons across segment 5 and 6 regions respectively while the remainder of the samples did not amplify. These samples were classified as 'untyped' and represented 25% and 27% of the samples respectively. As it is not known whether these samples represented novel HPR types which could not be typed using the existing method or whether they represented samples containing too little virus to type, these samples were grouped together and included in further analysis.

It should be noted that the data used in estimation in some cases include multiple cages from the same site. While this increases the sample size and the observed variation between ISAV strain and mortality rate, it also raises the possibility of correlation in mortality across cages within particular sites. However, controlling for site-specific effects with a set of indicator variables for the site in which the cage was located was not feasible owing to the relatively small number of cages per site in the data. Controls for bay management area will account for some of the potential correlation.

The segment 5 amplicons were sequenced and found to contain identical sequences across the variable region and were excluded from subsequent analyses. The HPR amplicons were sequenced and the translated amino acid sequence compared to known HPR types based on size and location of deletion (Nylund et al., 2003; Mjaaland et al., 2002). Three primary HPR types were found: HPR2, HPR4, and HPR0, along with several variants of the HPR 2 and 4 (i.e. HPR2.a and HPR2.b) which varied at one or more amino acids in the region (Figure 1). The HPR4 types were found at 9 different sites while the HPR2 types and HPR0 types were found at 3 and 8 different sites respectively. Untyped samples described above originated from a further 14 sites. In no cases were different HPR types identified from fish in the same cage, however fish typed as HPR2 and HPR4 were isolated from the same site, and several Untyped fish were found in sites containing HPR2 and HPR0 fish.

STATISTICAL ANALYSES

The relationship between mortality, measured as the logarithm of the mortality rate and ISAV molecular type was determined by regression analysis. Regression results give the estimated impact of the specific variable of interest on mortality holding other included factors constant. The results of the regression analyses were used to generate predictions of mortality levels and mortality rates for the range of specifications considered. These predictions are illustrated in Figure 2 and the associated values and p-values are shown in Table 1.

The first set of results (Model 1) compares mortality with ISAV type only, with Untyped ISAV, HPR0, HPR2, and HPR4 considered. In Figure 2 it can be clearly seen that cages where Untyped ISAV was detected, and where either HPR0 or HPR2 was detected, have markedly lower mortality rates than in cages where HPR4 was detected. The corresponding columns of Table 1 indicate that the mortality rate for HPR4 is statistically significantly lower than for HPR0, the reference group (p-value = 0.000). The second set of results (Model 2) is based on a specification that controls for the effects of year class and for certain events that had a significant impact on mortality but were unrelated to ISAV.[3] Controlling for these factors, mortality rates in cages with untyped ISAV are higher than in Model 1 but are still less than mortality rates from HPR4. However, from Table 1a it can be seen that the mortality rates in cages with HPR4 are no longer significantly higher than when HPR0 is present (p-value = 0.067) and this is also the case for HPR2 and untyped ISAV. Model 3a adds a crude location control for broad geography: whether the site is located in the Northern part or Southern part of the Bay of Fundy. Again there is little impact on the predicted mortality rates by ISAV type and in particular, differences in mortality rates across ISAV types are not statistically significant (e.g., p-value between HPR0 and HPR4 is 0.254). Model 3b replaces the location control with a set of controls for the Bay Management Area (BMA) at which the site is located (there are four represented in the data). The results are striking: mortality rates for HPR2 are predicted to surge to more than 3%, an unrealistic figure and one that is markedly above the predicted mortality rate for HPR4. The result arises because HPR2 is found to have been present only in cages and sites in bay management area 1, so that identification of HPR2 effects separately from cofounding factors related to bay management area is difficult. This serves to highlight the importance of large sample sizes to overcome such identification problems.

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[3] Specifically, a variable was included to reflect the occurrence of seal attacks that caused significant mortality in certain cages during the sampling period. In results not reported here, other variables were also included to reflect mortality caused by BKD and by skin lesions arising from poor weather. The inclusion of these variables had little effect on any of the results reported in the tables and figures.

DISCUSSION

We have investigated the relationship between mortality in the field and infection with different ISAV molecular types. The study represents a snapshot of ISAV infection and mortality in the Bay of Fundy during the winter 2005/06, and is to our knowledge the first field study of its kind. Our study included 62 ISAV positive cages on 17 sites. Fish in these cages were infected with one of three different ISAV HPR types (HPR0, HPR2 and HPR4) and all fish contained the same sequence across the variable region of segment 5.

Approximately 25% of fish could not be typed at either ISAV locus. This is likely due to low viral titre rather than failure of the typing assay. However, on the off chance that this was not the case the segment 6 untyped samples were included in the statistical analysis. Interestingly, all segment 6 untyped positives originated from the 2005 year class suggesting that the viral infectivity was relatively recent or, if the untyped positives represented a new molecular type which could not be detected, that the new molecular type had yet to become established in the population. This was generally believed to be the case with HPR0 when it was first identified, and it will be important to keep monitoring these ISAV positive/HPR 'negative' samples in future.

Both HPR4 and HPR0 types seemed to be widespread (i.e. present in non- adjacent zones), whereas HPR2 types seemed more localized. However, as just three sites were infected with this HPR, additional data will be needed to confirm this observation. One site housed infections of HPR2 and HPR4 types simultaneously, but no cages were found to contain more than one HPR type. Fish stocked in 2004 were infected with all three HPR type; however, the HPR0 was not found in fish stocked in 2005. Interestingly, HPR types found previous to 2004 (ie. HPR3 and HPR6) were not seen, yet HPR4, detected as far back as 1998 persists. All isolates in this 3 month survey other than HPR0, were shown by sequencing to be 'North American' and are considered virulent. European ISAV isolates identified previously such as HPR2 (European), and HPR8 (European) (R.Ritchie, personal communication) were not found.

Anecdotal field data has on many occasions indicated HPR4 types as virulent, HPR2 types as much less virulent, and HPR0 as likely avirulent. We find what appear to be large and significant differences in fish mortality by type of ISAV present which agree with these anecdotal findings. However, after the inclusion of control variables for confounding factors related to time in the cage

and location of the site, these differences are no longer statistically significant at conventional levels. Further, predicted mortality rates for the less virulent HPR2 are estimated to be very sensitive to how controls for location effects are specified. While our mortality data are from a relatively small number of cages and sites, the results do suggest that caution is warranted in concluding that mortality rates are significantly affected by ISAV type until other possible confounding factors have been accounted for. This will likely require much larger samples collected over many areas in order to be more confident that location-specific factors are not driving observed differences in mortality by ISAV strain.

This study was noteworthy in that it suggests that there may indeed be an association between HPR type and ISA mortality in the field. However, our study like others was limited by the field information we were able to gather, and our findings would benefit from a more in depth study. Our study, like others, focused on moribund fish or fish collected through routine surveillance of suspect sites, and we have likely under-represented the incidence and mortality associated with lower virulent types as these types are more likely to be associated with seemingly healthy fish. Indeed in samples where ISAV segment 6 was untyped, associated mortalities were much lower than with HPR4 infected fish (see Figure 2). This may have affected the p-values obtained, since splitting the sample in this manner is likely to increase standard errors, leading to larger confidence intervals. Future studies should aim to include representative samples of fish from infected and apparently uninfected cages. This will provide important mortality data for ISAV negative cages which will allow us to control for non-ISAV mortality more accurately.

While our work suggests an association between molecular type and virulence we have not attempted to address the molecular basis for this. It appears likely that virulence determinants exist on segment 6; however, further research will be required to elucidate the mechanism behind this association. Similarly, variation on segment 5, although not observed in this study, has been found in North American and European isolates (Devold 2006; and Ritchie personal communication), and by analogy with human influenza, certainly warrants further study.

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TABLES

Table 1: Log mortality rate – estimated impact of HPR type

	Model 1 estimate	p-value	**Model 2** estimate	p-value	**Model 3a** estimate	p-value	**Model 3b** estimate	p-value
HPR0	0.106	n/a	0.273	n/a	0.276	n/a	0.117	n/a
HPR2	0.270	0.286	0.311	0.915	0.310	0.904	3.108	0.249
HPR4	0.899	0.000	0.748	0.067	0.917	0.254	1.012	0.000
untyped	0.271	0.140	0.599	0.440	0.578	0.434	0.232	0.437

FIGURES

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          308                                                        374
Hpr0        SLGNTDTLIMREVALHKEMISKLQRNITDVKIRVDAIPPQLNQTFNTNQVEQPSTSVLSNIFISMGV
Hpr2        G......Q....LEAQ....G..G......NN..........--------------------MGV
Hpr2.a      G......Q....LEAQ.G..G..G......NN..........--------------------MGV
Hpr2.b      G......Q....LKAQ....G..G......NN..........--------------------MGV
Hpr4        G......Q....LEAQ....G..G......NN........----------------SNIFISMGV
Hpr4.a      G......Q....LEAQ....G..G......NN..A.....----------------SNIFISMGV
Hpr4.b      G......Q....LEAQ....G..G...A..NN........----------------SNIFISMGV
Hpr4.c      G......Q....LEAQ....G..G.T....NN........----------------SNIFISMGV
Hpr4.d      G......H....LEAQ....G..G...A..NN........----------------SNIFISMGV
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Fig. 1 Amino acid sequence alignment of Hpr region on Segment 6 of ISAV isolates found in the Bay Fundy in winter 2006. Dots represent conserved residues and dashes represent alignment gaps.

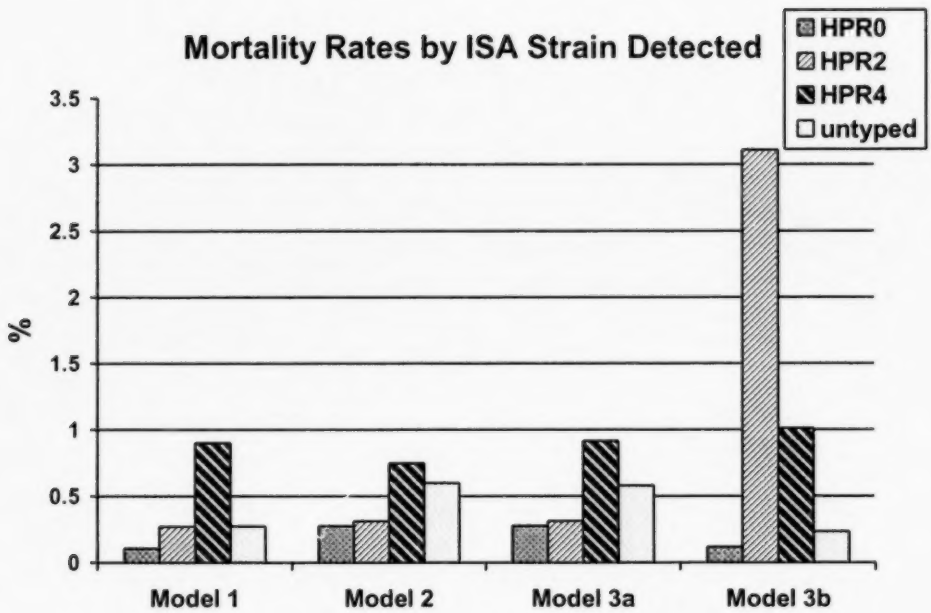

Figure 2: Graph of Mortality Rates under different statistical models
Mortality rates for each ISAV type are detailed in the legend. Model 1 controls for ISAV type; Model 2 controls for ISAV type, the effects of year class and for certain events that had a significant impact on mortality; Model 3a controls for ISAV type, the effects of year class, for certain events that had a significant impact on mortality control, and for approximate geographic location; Model 3b controls for ISAV type, the effects of year class, for certain events that had a significant impact on mortality control, and for BMA.